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TOXICITY OF MODIFIED HL SIMULANT AND METHYL SALICYLATE IN SOIL ON CUCUMBERS AND EARTHWORMS

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Tests were conducted to determine if methyl salicylate (MS), a component of HL simulant, was responsible for the toxicity exhibited by cucumbers and earthworms. The HL simulant without methyl salicylate (HLMS) and MS were tested for their toxicity to cucumbers and earthworms. The HLMS was tested at 0, 1,000, 5,000, 10,000, and 25,000 mg/kg by weight; MS was tested at 0, 50, 100, 200, 350, and 500 mg/kg by weight. The HLMS in soil produced no lethal or sublethal effects on cucumber plants at 1,000, 5,000, and 10,000 mg/kg levels, but the HLMS in soil produced sublethal effects at the 25,000 mg/kg level. Earthworms treated with HLMS had a survival rate of 100% for all treatment levels. The MS produced sublethal effects on cucumbers at the 350 and 500 mg/kg levels. Earthworms in soil dosed with MS showed an increasing weight loss with increasing concentrations. Survival rates were 100% at the 0-200 mg/kg levels, 87% at 350 mg/kg, and 0% at the 500 mg/kg level. An earlier CRDEC study (CRDEC-TR-257, March 1991), HL simulant containing 130 mg/kg of MS produced lethal and sublethal effects on earthworms and cucumbers. The study results indicated that MS is responsible for the effects exhibited by cucumbers and earthworms.

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PREFACE

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QUALITY ASSURANCE

This study was examined for compliance with Good Laboratory Practices as published by the U. S. Environmental Protection Agency in 40 CFR Part 792 (effective 18 September 1989). The dates of all inspections and the dates the results of those inspections were reported to the Study Director and management were as follows:

Phase Inspected	Date Inspected	Date Reported
Preparation of soil stock.	24 September 1990	28 September 1990
Earthworm weighing and placement in soil.	25 September 1990	28 September 1990

To the best of my knowledge, the methods described in this report were the methods followed during the study as indicated by the raw data found in the laboratory notebook. The report was determined to be an accurate reflection of the raw data recorded.

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Quality Specialist, Life Sciences Office

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TOXICITY OF MODIFIED HL SIMULANT AND METHYL SALICYLATE IN SOIL ON CUCUMBERS AND EARTHWORMS

1. INTRODUCTION

Persistent chemical agent simulants (PCAS) mimic blister and nerve agents that are used in field-training exercises. Simulants are needed to replace hazardous agents during these exercises and be environmentally safe to use.

An earlier study[1] indicated that the HL training simulant may cause adverse environmental effects if the field release level was at or near 1,000 mg/kg (by weight) in soil. The purpose of this study was to determine if methyl salicylate (MS), a component comprising 13% of the HL simulant mixture, was causing the toxic effects exhibited by cucumbers and earthworms. The study consisted of testing cucumber plants and earthworms with either HL simulant without MS (HLMS) or with MS alone incorporated into the growing medium. This would then provide a basis on which alteration of the HL simulant formula may be made to develop an environmentally safe compound and an effective training simulant. The data generated will be used in environmental documents and assist decision makers in amending the composition of this compound.

Data on the toxicity of chemical substances and mixtures are subject to environmental effects test regulations under the Toxic Substances Control Act (TSCA) (Pub. L. 94-469, 90 Stat. 2003, 14 U.S.C. 2601 et. seq.). Also, Army Regulation (AR) 200-2 integrates environmental considerations into Army plans and programs and provides the Army's requirements for documentation of environmental matters.[2] Phytotoxicity data are used to evaluate effects of compounds on plants that serve as the primary source of food, shelter, and biomass for virtually all natural communities. The type of phytotoxicity testing employed in this study uses plant height and biomass measurements as plant growth indicators.

The second phase of this study involved earthworm toxicity testing. Earthworms, because of their role in maintaining the physical characteristics and processes of soil, such as aeration, water permeability, and breakdown of organic matter, are considered key organisms in the soil community. Earthworms, which can number up to 250,000 individuals per acre, increase the fertility of soil by increasing the availability of nutrients and are also an important link in the food chain. Roberts and Dorough[3] published a review of the importance of earthworms to terrestrial ecosystems and their use in assessing the hazards of chemicals to these nontarget organisms. Heimbach[4] compared laboratory methods using *Eisenia foetida* and *Lumbricus terrestris* to assess the hazards of chemicals to earthworms in an artificial soil. Based on this review, Heimbach recommended that *Eisenia foetida* be used as a representative species to assess the toxicity of chemicals to earthworms. A well-defined artificial soil substrate is recommended for standardized earthworm toxicity tests.[5,6]

2. METHODS AND MATERIALS

2.1 Test Chemicals.

2.1.1 HLMS.

The HL simulant was developed to mimic the physical properties of the mustard-Lewisite agent and to be used during field-training exercises. The first phase of this study involved altering the present composition of HL simulant by eliminating the MS during mixture preparation. The MS was replaced with an equal amount of distilled water to retain the current percentages of the other components (Table 1).

Table 1. Characteristics of HL Simulant

Constituent	Function	Percentage (by weight)
Ferrous Ammonium Sulphate	Provides Fe^{++} Cue	1.98
Polyethylene Oxide	Viscoelastic Thickener	0.25
Hydroxy Ethyl Cellulose	Stabilizer & "Scrubbability"	0.35
Glycerol	Plasticizer	9.91
Methyl Salicylate	Chemical Trigger	13.00
Water	Solvent and Extender	74.51

The other components (i.e., polyethylene oxide, hydroxy ethyl cellulose, glycerol, ferrous ammonium sulphate, and water) are relatively inert and, therefore, should not cause any of the exhibited toxic effects.

2.1.2 MS.

Methyl salicylate is an organic ester that can undergo acidic and alkaline hydrolysis of the ester to produce methyl alcohol and salicylic acid or a salicylic acid salt. Methyl salicylate and salicylic acid can undergo further biodegradation by aerobic and anaerobic microorganisms.

The objective of this study was to determine the acute environmental effects of MS on cucumber plants and earthworms.

2.2 Plant Study.

The test methods used for phytotoxicity studies were adapted from the U.S. Environmental Protection Agency's (USEPA) Environmental Effects Test Guidelines (Early Seedling Growth Toxicity Test).[7] The plant species selected for use in this study, chosen from the USEPA's list of recommended crops, was cucumber (Cucumis sativus L., cv. Straight Eight). Cucumber seeds were obtained from the Meyer Seed Company (Baltimore, MD) and sorted to ensure uniform size, with damaged and malformed seeds being discarded.

The soil used for these tests was obtained from a site at M-Field (APG-EA, MD). The soil was a moderately eroded acidic Sassafras sandy loam (fine-loamy, siliceous, mesic Typic Hapludult), yielding a loamy sand texture. The respective physical and chemical properties of the soil are given in Table 2.

Table 2. Physical and Chemical Characteristics of M-Field Soil

Soil Parameters*		
Mechanical Analysis	Soil Analysis	
% sand	NO_3 (Lb/A) -	9.2
87	P_2O_5 (Lb/A) -	14
	K_2O (Lb/A) -	48
% silt	Ca (Lb/A) -	10
9	Mg (Lb/A) -	24
	Mn (Lb/A) -	5
% clay	Zn (Lb/A) -	2
4	Cu (Lb/A) -	1.8
	CEC (meq/100 g) -	2.2
% organic matter - 0.3 Texture - sandy loam	pH -	4.6

*Determined by the Soil Testing Laboratory, University of Maryland, College Park, MD

The soil was air-dried and seived to pass a 2-mm screen. The test solution was mixed with a small amount of M-Field soil (on a weight basis) to produce a spike. The spike was mixed with additional M-Field soil to achieve the desired concentration. The concentrations used for dosing plants with HLMS were 0, 1,000, 5,000, 10,000, and 25,000 mg/kg by weight (the same concentrations used in the HL simulant study, which corresponds to 0, 130, 650, 1300, and 3250 mg/kg of MS in the simulant mixture, respectively). The concentrations of MS used for dosing plants were 0, 50, 100, 200, 350, and 500 mg/kg by weight.

In each experiment, individual treatment pots were prepared in triplicate for each concentration used. Treatment pots were prepared by placing a 10-cm^2 piece of cheesecloth in the bottom of each 15-cm flower pot, followed by 1300 g pea gravel (3 to 5-mm diameter). A second piece of moistened cheesecloth was placed on top of the pea gravel. The spiked soil/soil mixture (Table 3 for HLMS and Table 4 for MS) was first mixed in a food blender and then added to the pots.

Table 3. Experimental Treatments for HLMS on Plants

Test Concentration (mg/kg)	Flower Pot No.	Spiked Soil:Soil (g)
0 (control)	1, 2, 3*	0:1000
1,000	4, 5, 6	1:999
5,000	7, 8, 9	5:995
10,000	10, 11, 12	10:990
25,000	13, 14, 15	25:975

*Replicates in rows (e.g., 1, 2, 3); blocks in columns (e.g., 1, 4, 7, 10, 13); pots randomized within each block.

Table 4. Experimental Treatments for MS on Plants

Test Concentration (mg/kg)	Flower Pot No.	Spiked Soil:Soil (g)
0 (control)	1, 2, 3*	0:1000
50	4, 5, 6	2.5:997.5
100	7, 8, 9	5.0:995.0
200	10, 11, 12	10.0:990.0
350	13, 14, 15	17.5:982.5
500	16, 17, 18	25.0:975.0

*Replicates in rows (e.g., 1, 2, 3); blocks in columns (e.g., 1, 4, 7, 10, 13, 16); pots randomized within each block.

Twenty seeds per pot were planted to a depth of 5 mm. Pots were watered on a weight basis to provide 18% moisture. The experimental design for the plant study was a complete randomized block design with blocks of treatments replicated in triplicate.

Individual treatment pots within blocks were randomized once per week for the 14-day growth period. The pots within each block were rotated 180° three times per week because of cucumber plants' phototropic response to the sun.

After 50% of the control seedlings had germinated, the plants were thinned to the 10 most uniform plants in each pot. The plants were grown for 14 days following emergence (Day 1). Plant heights were measured in situ twice per week following thinning, and a final measurement was made at harvest on Day 14.

Biomass measurements for the fresh weights (fw) and dry weights (dw) were made at the end of the 14-day study. The plants from each pot were cut at the soil surface and weighed to obtain the fw for each dose level. The plants were placed in a drying oven for 3 days at 65 °C. At the end of 3 days, the dw were taken. The water content for each group of plants was determined by the following method:

$$\text{fw-dw/fw} \times 100 = \%\ \text{water}$$

Plant data were statistically analyzed using the two-way analysis of variance (ANOVA)[8] and the Newman-Keuls pairwise comparison of means[9] for plant heights. An ANOVA and the Newman-Keuls pairwise comparison of means were also used to analyze biomass measurements.

2.3 Earthworm Study.

The method to determine the toxicity of contaminants in soil to the earthworm, developed by Neuhauser et al.,[10] was adapted to determine the toxicity of HLMS and MS in soil. The earthworms used in the experiments were *Eisenia foetida*, purchased from Bert's Bait (Irvine, KY). The earthworms were housed in an incubator (13.0 °C, $\pm$0.2) for at least 2 weeks prior to testing.

The test media consisted of a nonsterile artificial soil (AS) and distilled water. The use of an AS limits test variability that would otherwise occur due to heterogeneity of soil parameters. Other advantages of using an AS mixture are ease of preparation and comparability to other data in the literature.[11, 12, 13] The components of AS used in the earthworm toxicity test were finely ground sphagnum peat (10% by weight), kaolinite clay (20%), fine sand (69%), and calcium carbonate (1%).

For each replicate, 200 g of AS was used. The HLMS was mixed with distilled water on a weight basis to provide the concentration needed to dose the AS (Table 5). The water solution and AS were thoroughly mixed in a food blender for approximately 3 min. The soil mixture was then added to a 600-mL beaker.

Table 5. Experimental Treatments for HLMS on Earthworms

Test Concentration (mg/kg)	Beaker #	AS (g)	Simulant:Water (mL)
0 (control)	1, 2, 3*	200.0	0:50.0
1,000	4, 5, 6	200.0	0.2:49.8
5,000	7, 8, 9	200.0	1.0:49.0
10,000	10, 11, 12	200.0	2.0:48.0
25,000	13, 14, 15	200.0	5.0:45.0

*Replicates in rows (e.g., 1, 2, 3); blocks in columns (e.g.,1, 4, 7, 10, 13); beakers randomized within each block.

The MS, which is only slightly soluble in water, was mixed with AS on a weight basis to provide a spike. The spike was then mixed with an additional amount of AS to obtain the desired concentration of test chemical. The spike/soil mixture was mixed in a food blender for 3 min until uniformly mixed. During mixing, 50 mL of distilled water was added to each 200 g batch of soil to provide a moisture level of 25%. The concentrations used for the earthworm study were the same as in the plant study (i.e., 0, 50, 100, 200, 350, and 500 mg/kg). The experimental design (Table 6) was a randomized complete block.

Table 6. Experimental Treatments for MS on Earthworms

Test Concentration (mg/kg)	Beaker #	Spiked Soil:Soil (g)
0 (control)	1, 2, 3*	0:200
50	4, 5, 6	0.5:199.5
100	7, 8, 9	1.0:199.0
200	10, 11, 12	2.0:198.0
350	13, 14, 15	3.5:196.5
500	16, 17, 18	5.0:195.0

*Replicates in rows (e.g., 1, 2, 3); blocks in columns (e.g., 1, 4, 7, 10, 13, 16); beakers randomized within each block.

Five earthworms were weighed as a group and groups were randomly added to each beaker. Three replicates per concentration were used for each experiment. Beakers were covered with nylon screen and cheesecloth, which was held in place by rubber bands. The beakers were randomly placed in a lowtemperature incubator at 13.0 °C, ± 0.2. After 14 days, the earthworms in each beaker were re-weighed and examined for physical condition.

The statistical methods used to evaluate earthworm data were the t-Test and the analysis of covariance (ANCOVA)[14] to test the weight differences of the earthworms.

3. RESULTS

3.1 Effects of HLMS on Plants.

The heights of the cucumber plants treated with HLMS are given in Appendix A, Table A-1. An ANOVA of the plant heights indicated a highly significant ($p < 0.01$) difference between treatments with no significant ($p > 0.05$) difference between blocks (Appendix B, Table B-1). A Newman-Keuls test was performed to determine whether these differences were due to treatment or other factors. The test showed that there was (1) a highly significant ($p < 0.01$) difference between the 1,000 mg/kg level, with enhanced growth, and all other levels tested; (2) a highly significant ($p < 0.01$) difference between the 25,000 mg/kg level, with reduced growth, and all other levels; and (3) a highly significant ($p < 0.01$) difference between the 5,000 and 10,000 mg/kg levels (Appendix B, Table B-2).

An ANOVA of the fw (Appendix B, Table B-3) and dw (Appendix B, Table B-4) indicated a highly significant ($p < 0.01$) difference between treatments for both of these variables. A Newman-Keuls pairwise comparison of means for the fw (Appendix B, Table B-5) and dw (Appendix B, Table B-6) indicated a highly significant ($p < 0.01$) difference between the 25,000 mg/kg level and all other levels tested. There was also a significant ($p < 0.05$) difference between dw at 1,000 mg/kg level and the other levels (i.e., 0, 5,000, and 10,000 mg/kg). The percentage of water in the plants at different treatment levels ranged from 88% at the 25,000 mg/kg level to 92% at the 5,000 mg/kg level.

Survival rates for cucumber plants grown in soil amended with the HLMS was 100% at all concentrations tested.

3.2 Effects of MS on Plants.

The heights of the cucumber plants treated with MS are given in Appendix A, Table A-2. An ANOVA of the plant heights indicated a highly significant ($p < 0.01$) difference between treatments with no significant ($p > 0.05$) difference between blocks (Appendix B, Table B-7). A Newman-Keuls test was performed to determine whether these differences were due to treatment or other factors. The test showed that there was a highly significant ($p < 0.01$) difference between the 350 and 500 mg/kg levels and the other levels tested (Appendix B, Table B-8).

The ANOVA of the fw showed a highly significant ($p < 0.01$) difference between treatments with no significant ($p > 0.05$) difference between blocks (Appendix B, Table B-9). The Newman-Keuls pairwise comparison of means for the fw showed a significant ($p < 0.05$) difference between the 500 mg/kg level and the other levels tested (Appendix B, Table B-10).

The ANOVA of the dw (Appendix B, Table B-11) was similar to the results of the fw. A Newman-Keuls test of the dw showed a highly significant ($p < 0.01$) difference between the 500 mg/kg level and the other levels (except for the 350 mg/kg level where the significance level was at ($p < 0.05$). There was also a significant ($p < 0.05$) difference between the 350 mg/kg level and the other levels tested (i.e., 0, 50, 100, and 200 mg/kg) (Appendix B, Table B-12).

The percentage of water in the plants at different treatment levels was 88% for the 50, 100, and 200 mg/kg levels, 89% for the 350 mg/kg level, and 90% for the 0 and 500 mg/kg levels.

Survival rates for cucumber plants grown in soil amended with MS was 100% at all concentrations tested.

3.3 Effects of HLMS on Earthworms.

The results of the HLMS test showed no lethal or sublethal (e.g., a significant weight loss over the 14-day study period as compared to controls) effects on earthworms at the concentrations used in this study. The earthworms at each treatment level gained weight (Appendix C, Table C-1) and had a 100% survival rate. The ANCOVA (Appendix D, Table D-1) of earthworm weights showed that the difference between the initial weights and the final weights was not significant ($p > 0.05$) among the various treatment levels. In addition, the t-test showed no significant ($p > 0.05$) difference for the weight differences (Appendix, Table D-2).

3.4 Effects of MS on Earthworms.

The results of this test showed that as the concentration of MS in soil increased, there was a correspondingly larger weight loss and a decrease in survival rates. Survival rates were 100% for the controls through the 200 mg/kg level. The survival rate was 87% for the 350 mg/kg level and 0% for the 500 mg/kg level.

The MS produced no lethal effects on earthworms up to the 200 mg/kg level. However, earthworms lost weight at the 100 mg/kg level. This weight loss continued up to the 350 mg/kg level with no earthworms surviving at the 500 mg/kg level (Appendix C, Table C-2). The ANCOVA (Appendix D, Table D-3) statistical analysis of the weight change data for earthworms indicated a significant ($p < 0.05$) difference between treatments through the 350 mg/kg level. The t-test indicated a highly significant ($p < 0.01$) difference between the 350 mg/kg level and the control group (i.e., 0 mg/kg) (Appendix D, Table D-4). There was also a significant ($p < 0.05$) difference between the 350 mg/kg level and the other concentrations tested (i.e., 50, 100, and 200 mg/kg). This indicates that acute earthworm toxicity begins at a concentration between 350 and 500 mg/kg.

4. DISCUSSION

The HLMS produced no lethal effects on cucumber plants through the 25,000 mg/kg level. Some growth enhancement was observed at the 1,000 mg/kg level, in which case the components of HLMS may be providing additional nutrients. However, a sublethal effect was observed at the highest dose level (i.e., 25,000 mg/kg). In this case, the plants were significantly shorter than the controls, thus indicating that HLMS was toxic to plants at higher concentrations. Similar effects have been observed when plants were given too much fertilizer.

When the results of the HLMS study were compared to the HL simulant study, it was found that HLMS did not produce toxic effects until the concentration reached the 25,000 mg/kg level. However, the HL simulant produced lethal and sublethal effects on cucumbers at the 1,000 mg/kg level (Table 7).

Table 7. Comparison of HLMS and HL Simulant Studies on Cucumbers

	Concentration Levels (mg/kg)				
Parameters	0	1,000	5,000	10,000	25,000
Survival Rates (%):					
HLMS	100	100	100	100	100
HL Simulant	100	100	0	0	0
Av. Plant Heights (mm):					
HLMS	46	54	49	44	27
HL Simulant	52	35	-	-	-
Av. FW (g):					
HLMS	0.46	0.55	0.49	0.45	0.24
HL Simulant	0.52	0.38	-	-	-
Av. DW (g):					
HLMS	0.04	0.05	0.04	0.04	0.03
HL Simulant	0.04	0.03	-	-	-
% Water:					
HLMS	91	91	92	91	88
HL Simulant	92	92	-	-	-

The HLMS in soil produced neither lethal nor sublethal effects on earthworms. There was a 100% survival rate and an overall weight gain at all levels tested. This indicates that HLMS should not produce any harmful effects to the environment at field-release levels of 10 g/m^2.

The HL simulant mixture was more toxic to cucumbers and earthworms at lower concentrations than either HLMS or MS. The HLMS produced sublethal effects on cucumbers only at the highest concentration tested (i.e., 25,000 mg/kg); whereas, the HL simulant produced sublethal effects at the 1,000 mg/kg level.

All of the earthworms survived at all concentrations tested in the HLMS study, in which no MS was incorporated into the mixture. The earthworm survival rate for the HL simulant was 20% at the 1,000 mg/kg (130 mg/kg of MS in the mixture). However, the survival rate was 100% for MS at the 200 mg/kg level and 87% at 350 mg/kg level. None of the earthworms survived at the 500 mg/kg level, which is comparable to the results of the HL simulant study where none of the earthworms survived at the 5,000 mg/kg level (650 mg/kg of MS).

A comparison was made between survival rates of earthworms dosed with HLMS in this study and those dosed with the original HL simulant from an earlier study.[1] This comparison showed that the HL simulant without MS produced no lethal or sublethal effects on earthworms at the concentrations tested, but the original HL simulant produced lethal and sublethal effects at the 1,000 mg/kg level.

The MS was found to be toxic to vegetation at 1 to 2 $\mu g/cm^2$ when applied to foliar surfaces via an aerosol application.[15] In the present study, MS, which was incorporated into the planting medium, did not produce any detrimental effects on cucumber plants until the MS level reached 350 mg/kg. The mean plant heights of cucumbers at the 100 mg/kg level were significantly ($p < 0.05$) taller than the controls. However, the HL simulant produced sublethal effects on cucumbers at the 1,000 mg/kg level, which has 130 mg/kg of MS in the mixture.

Methyl salicylate produced sublethal effects on earthworms (i.e., an average weight loss as compared to controls) at 100 mg/kg. This is comparable to the HL simulant study in which lethal effects were produced at 130 mg/kg level of MS (1,000 mg/kg level of HL simulant). However, MS was not as toxic as the original HL simulant mixture. There was a 100% survival rate at 200 mg/kg and an 87% survival rate at 350 mg/kg for MS. In a previous study,[1] the HL simulant produced only a 20% survival rate at the 130 mg/kg level with no survivors at the 650 mg/kg level. The increased toxicity of the HL simulant could be due to a synergistic effect produced by the combination of components in the mixture.

Methyl salicylate was found to be lethal to earthworms via an aerosol dose of 463 mg/kg with a survival rate of 86%.[15] Again, this is similar to the results of this study in which there was an earthworm survival rate of 87% at 350 mg/kg and 0% at 500 mg/kg of MS in soil.

5. CONCLUSION

It is suggested that methyl salicylate in the simulant formula caused the lethal and sublethal effects exhibited by earthworms and cucumber plants. The results of these tests coupled with the results from the HL simulant study indicated that the other components of the simulant formula have a negligible impact on cucumbers and earthworm. It is recommended that additional research be conducted to determine if a lower concentration of MS (e.g., 50 mg/kg) will still activate the chemical agent disclosure solution. This may then provide an effective training agent and an environmentally safe simulant.

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APPENDIX A

PLANT DATA

Heights of Cucumber Plants Grown in Soil Amended with Either HLMS or MS

Table A-1. Heights of Cucumber Plants on Day 14, Grown In Soil Amended with HLMS

Replicate No.	Plant No.	Concentrations (mg/kg) 0	1,000	5,000	10,000	25,000
		plant heights (mm)				
I	1.	51	55	47	37	27
	2.	40	43	53	39	26
	3.	39	60	48	39	27
	4.	59	41	38	43	35
	5.	56	52	52	45	29
	6.	53	49	55	49	33
	7.	44	68	52	42	25
	8.	42	47	53	46	24
	9.	36	57	41	54	25
	10.	43	58	39	42	21
II	1.	38	41	49	39	32
	2.	44	48	46	41	31
	3.	47	52	37	43	25
	4.	51	47	40	42	29
	5.	36	56	47	37	28
	6.	40	67	48	42	26
	7.	33	62	55	48	27
	8.	38	60	46	37	26
	9.	39	67	45	43	21
	10.	37	38	38	45	29
III	1.	49	56	59	44	28
	2.	60	47	61	54	35
	3.	54	64	46	47	37
	4.	48	69	51	44	27
	5.	51	40	57	35	34
	6.	59	60	57	47	22
	7.	46	56	48	54	21
	8.	52	59	51	48	20
	9.	45	51	60	47	23
	10.	52	69	53	32	29
Mean:		46.1	54.6	49.0	43.5	27.4
Std. Dev.:		7.6	9.2	6.8	5.4	4.5

Table A-2. Heights of Cucumber Plants on Day 14, Grown In Soil Amended with MS

Replicate No.	Plant No.	Concentrations (mg/kg) 0	50	100	200	350	500
		plant heights (mm)					
I	1.	28	29	31	31	26	22
	2.	29	28	42	29	27	23
	3.	28	35	34	32	23	21
	4.	33	43	35	33	32	21
	5.	34	35	42	32	23	36
	6.	31	32	41	30	31	24
	7.	30	30	35	32	21	17
	8.	30	32	37	30	22	20
	9.	34	32	31	31	22	17
	10.	32	34	33	40	21	5
II	1.	29	27	33	31	30	16
	2.	28	39	30	36	25	20
	3.	35	34	35	38	18	18
	4.	33	31	39	35	28	5
	5.	31	30	39	29	25	27
	6.	29	42	38	36	28	24
	7.	25	32	32	32	29	18
	8.	30	30	40	32	26	15
	9.	29	33	42	33	30	
	10.	26	33	37	32	25	17
III	1.	35	30	35	37	33	19
	2.	35	33	37	40	32	17
	3.	39	39	29	37	18	18
	4.	34	43	28	39	25	9
	5.	42	38	34	39	25	3
	6.	42	38	45	43	19	5
	7.	37	45	33	37	28	4
	8.	32	35	35	36	33	7
	9.	36	40	34	36	17	5
	10.	33	35	42	40	21	8
Mean:		32.3	34.6	35.9	34.6	25.4	15.6
Std. Dev.:		4.2	4.8	4.4	3.8	4.7	8.1

APPENDIX B

STATISTICAL DATA - PLANTS

Analysis of Variance (ANOVA) of Heights of Cucumber Plants Grown in Soil Amended with Either HLMS or MS
and
Newman-Keuls Analysis of Treatment of Cucumber Plant Heights, Fresh Weights and Dry Weights Grown in Soil Amended with Either HLMS or MS

Table B-1. Analysis of Variance, Two-way, Single Observation: Plant Height (mm) of Cucumber (10 Plants/Block), Grown in Soil Amended with HLMS

Parameter	0	1,000	5,000	10,000	25,000
N:	30	30	30	30	30
Mean:	46.1	54.6	49.0	43.5	27.4
Std. Dev.	7.6	9.2	6.8	5.4	4.5

Source of Variation	Sum of Squares	Degrees of Freedom	Mean Square	F Value
Total:	19449.840	149		
Treatments:	12542.307	4	3135.5767	74.95
Blocks:	789.160	2	394.585	9.43
Within:	470.173	8	58.772	1.41

Differences Between Treatments: Significant at $p < 0.01$

Differences Between Blocks: Not Significant at $p < 0.05$

Table B-2. Newman-Keuls Analysis of All Treatments, Pairwise, and Ranked From Low to High: Cucumber Plant Heights (mm), Grown in Soil Amended with HLMS

Treatment	25,000	10,000	0	5,000	1,000
			q values		
25,000		12.776**	14.813**	17.141**	21.611**
10,000			2.036	4.364**	8.835**
0				2.327	6.798**
5,000					4.470**
q(95%)		2.77	3.31	3.63	3.86
q(99%)		3.64	4.12	4.4	4.6

** Significant at $p < 0.01$

Table B-3. Analysis of Variance, Two-way, Single Observation: Fresh Weight (g) of Cucumber Plants Grown in Soil Amended with HLMS

Parameter	0	1,000	5,000	10,000	25,000
N:	3	3	3	3	3
Mean:	4.62	5.45	4.87	4.45	2.36
Std. Dev:	0.40	0.42	0.19	0.22	0.10

Source of Variation	Sum of Squares	Degrees of Freedom	Mean Square	F Value
Total:	17.404	14		
Treatments:	16.543	4	4.1357	52.26
Blocks:	0.228	2	0.1139	1.44
Within:	0.633	8	0.0791	

Differences Between Treatments: Significant at $p < 0.01$

Differences Between Blocks: Not Significant at $p < 0.05$

Table B-4. Analysis of Variance, Two-way, Single Observation: Dry Weight (g) of Cucumber Plants Grown in Soil Amended with HLMS

Parameter	0	1,000	5,000	10,000	25,000
N:	3	3	3	3	3
Mean:	0.39	0.48	0.43	0.40	0.26
Std. Dev:	0.04	0.06	0.01	0.01	0.02

Source of Variation	Sum of Squares	Degrees of Freedom	Mean Square	F Value
Total:	8.716	14		
Treatments:	0.075	4	0.0188	31.26
Blocks:	0.007	2	0.0035	5.84
Within:	0.005	8	0.0006	

Differences Between Treatments: Significant at $p < 0.01$

Differences Between Blocks: Significant at $p < 0.05$

Table B-5. Newman-Keuls Analysis of All Treatments, Pairwise, and Ranked From Low to High: Cucumber Fresh Weights (g), Grown in Soil Amended with HLMS

Treatment	25,000	10,000	0	5,000	1,000
			q values		
25,000		12.338**	13.322**	14.817**	18.222**
10,000			0.983	2.479	5.883**
0				1.495	4.899*
5,000					3.404*
q(95%)		3.15	3.88	4.33	4.65
q(99%)		4.48	5.27	5.77	6.14

*Significant at $p < 0.05$
**Significant at $p < 0.01$

Table B-6. Newman-Keuls Analysis of All Treatments, Pairwise, and Ranked From Low to High: Cucumber Dry Weights (g), Grown in Soil Amended with HLMS

Treatment	25,000	0	10,000	5,000	1,000
			q values		
25,000		6.355**	6.791**	8.234**	10.766**
0			0.436	1.878	4.410*
10,000				1.442	3.974*
5,000					2.532*
q(95%)		3.15	3.88	4.33	4.65
q(99%)		4.48	5.27	5.77	6.14

*Significant at $p < 0.05$
**Significant at $p < 0.01$

Table B-7. Analysis of Variance, Two-way, Single Observation: Plant Height (mm) of Cucumber (10 Plants/Block), Grown in Soil Amended with MS

Parameter	0	50	100	200	350	500
N:	30	30	30	30	30	30
Mean:	32.3	34.6	35.9	34.6	25.4	15.6
Std. Dev:	4.2	4.8	4.4	3.8	4.7	8.1

Source of Variation	Sum of Squares	Degrees of Freedom	Mean Square	F Value
Total:	13992.728	179		
Treatments:	9309.4278	5	1861.8856	89.057
Blocks:	40.0778	2	20.0389	0.958
Within:	1256.3222	10	125.63222	6.009

Differences Between Treatments: Significant at $p < 0.01$

Differences Between Blocks: Not Significant at $p < 0.05$

Table B-8. Newman-Keuls Analysis of All Treatments, Pairwise, an Ranked From Low to High: Cucumber Plant Heights (mm), Grown in Soil Amended with MS

Treatment	500	350	0	50	200	100
				q values		
500		10.381**	17.630**	20.023**	20.059**	21.466**
350			7.249**	9.642**	9.677**	11.085**
0				2.393	2.428	3.835*
50					0.035	1.442
200						1.407
q(95%)		2.77	3.31	3.63	3.86	4.03
q(99%)		3.64	4.12	4.4	4.6	4.76

*Significant at $p < 0.05$
**Significant at $p < 0.01$

Table B-9. Analysis of Variance, Two-way, Single Observation: Fresh Weight (g) of Cucumber Plants Grown in Soil Amended with MS

Parameter	0	50	100	200	350	500
N:	3	3	3	3	3	3
Mean:	2.85	3.25	3.18	3.33	2.68	2.01
Std. Dev:	0.41	0.14	0.24	0.38	0.14	0.48

Source of Variation	Sum of Squares	Degrees of Freedom	Mean Square	F Value
Total:	4.924	17		
Treatments:	3.665	5	0.7330	7.54
Blocks:	0.287	2	0.1436	1.48
Within:	0.972	10	0.0971	

Differences Between Treatments: Significant at $p < 0.01$

Differences Between Blocks: Not Significant at $p < 0.05$

Table B-10. Newman-Keuls Analysis of All Treatments, Pairwise, and Ranked From Low to High: Cucumber Fresh Weights (g), Grown in Soil Amended with MS

Treatment	500	350	0	100	50	200
				q values		
500		3.587*	4.490*	6.232**	6.613**	7.057**
350			0.903	2.645	3.025	3.470
0				1.741	2.122	2.566
100					0.380	0.825
50						0.444
q(95%)		3.08	3.77	4.2	4.51	4.75
q(99%)		4.32	5.04	5.5	5.84	6.1

*Significant at $p < 0.05$
**Significant at $p < 0.01$

Table B-11. Analysis of Variance, Two-way, Single Observation: Dry Weight (g) of Cucumber Plants Grown in Soil Amended with MS

Parameter	0	50	100	200	350	500
N:	3	3	3	3	3	3
Mean:	0.35	0.37	0.37	0.35	0.28	0.20
Std. Dev:	0.04	0.02	0.02	0.03	0.01	0.05

Source of Variation	Sum of Squares	Degrees of Freedom	Mean Square	F Value
Total:	0.079	17		
Treatments:	0.067	5	0.0135	12.79
Blocks:	0.001	2	0.0006	0.59
Within:	0.011	10	0.0011	

Differences Between Treatments: Significant at $p < 0.01$

Differences Between Blocks: Not Significant at $p < 0.05$

Table B-12. Newman-Keuls Analysis of All Treatments, Pairwise, and Ranked From Low to High: Cucumber Dry Weights (g), Grown in Soil Amended with MS

Treatment	500	350	0	200	50	100
				q values		
500		4.077*	7.848**	8.319**	9.229**	9.332**
350			3.771*	4.241*	5.151*	5.254*
0				0.470	1.380	1.483
200					0.909	1.012
50						0.103
q(95%)		3.08	3.77	4.2	4.51	4.75
q(99%)		4.32	5.04	5.5	5.84	6.1

*Significant at $p < 0.05$
**Significant at $p < 0.01$

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APPENDIX C

EARTHWORM DATA

Weight Differences (±) of Earthworms in Soil Amended with Either HLMS or MS

Table C-1. Weight Differences (±) of Earthworms in Soil Amended with HLMS

Concen (mg/kg)	No. of initial earth-worms	Average initial weight (g)	No. of final earth-worms	Average final weight (g)	Mean initial weight (g)	Mean final weight (g)	Net weight change (±)
	5	0.33	5	0.37			
0	5	0.33	5	0.36	0.32	0.36	+0.04
	5	0.30	5	0.34			
	5	0.30	5	0.37			
1,000	5	0.29	5	0.34	0.29	0.35	+0.06
	5	0.28	5	0.33			
	5	0.34	5	0.37			
5,000	5	0.29	5	0.35	0.32	0.38	+0.06
	5	0.33	5	0.43			
	5	0.32	5	0.38			
10,000	5	0.29	5	0.35	0.30	0.36	+0.06
	5	0.30	5	0.34			
	5	0.28	5	0.32			
25,000	5	0.30	5	0.37	0.29	0.36	+0.07
	5	0.31	5	0.39			

Table C-2. Weight Differences ($\pm$) of Earthworms in Soil Amended with MS

Concen (mg/kg)	No. of initial earth-worms	Average initial weight (g)	No. of final earth-worms	Average final weight (g)	Mean initial weight (g)	Mean final weight (g)	Net weight change ($\pm$)
	5	0.35	5	0.36			
0	5	0.37	5	0.42	0.36	0.38	+0.02
	5	0.35	5	0.35			
	5	0.36	5	0.35			
50	5	0.31	5	0.31	0.34	0.35	+0.01
	5	0.37	5	0.38			
	5	0.33	5	0.31			
100	5	0.37	5	0.30	0.34	0.33	-0.01
	5	0.34	5	0.37			
	5	0.48	5	0.47			
200	5	0.33	5	0.31	0.39	0.38	-0.01
	5	0.36	5	0.35			
	5	0.36	4	0.29			
350	5	0.34	5	0.25	0.36	0.29	-0.07
	5	0.40	4	0.34			
	5	0.37	0	-			
500	5	0.31	0	-	0.35	-	-
	5	0.36	0	-			

APPENDIX D

STATISTICAL DATA - EARTHWORMS

Analysis of Covariance (ANCOVA) of Weight Differences of Earthworms Raised in Artificial Soil Amended with Either HLMS or MS
and
T-Test Analysis Among Adjusted Weight Means (g) of Earthworms Raised in Soil Amended with Either HLMS or MS

Table D-1. ANCOVA of Weight Differences (g) of Earthworms in Soil Amended with HLMS

Source of Variation	Sum of Squares	Degrees of Freedom	Mean Squares	F Value	Significance Level
Treatment Levels	0.00155	4	0.00038	0.91	0.4957*

*Not significant at $p > 0.05$

Table D-2. T-Test Analysis of All Treatments: Final Weights (g) of Earthworms Raised in Soil Amended with HLMS

Treatment	0	10,000	1,000	5,000	25,000
			p values		
0		0.2961	0.2326	0.1473	0.1336
10,000	0.2961		0.7446	0.7125	0.5237
1,000	0.2326	0.7446		0.9644	0.7584
5,000	0.1473	0.7125	0.9644		0.8182
25,000	0.1336	0.5237	0.7584	0.8182	

*Not Significant at $p < 0.05$

Table D-3. ANCOVA of Weight Differences (g) of Earthworms in Soil Amended with MS

Source of Variation	Sum of Squares	Degrees of Freedom	Mean Squares	F Value	Significance Level
Treatment Levels	0.01453	4	0.00363	4.60	0.0268*

*Not significant at $p > 0.05$

Table D-4. T-Test Analysis of All Treatments: Final Weights (g) of Earthworms Raised in Soil Amended with MS

Treatment	0	50	100	200	350
			p values		
0		0.4165	0.1196	0.1834	0.0028**
50	0.4165		0.4061	0.5596	0.0113*
100	0.1196	0.4061		0.8432	0.0456*
200	0.1834	0.5596	0.8432		0.0328*
350	0.0028	0.0113	0.0456	0.0328	

*Significant at $p < 0.05$
**Significant at $p < 0.01$